REMARKS

These Remarks are provided in response to the comments attached to the Advisory Action mailed January 16, 1998, for parent application Serial No. 08/571,802.

I. Provisional Obviousness-type Double Patenting Rejection

Claims 1-2, 8-9 and 15-16 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being allegedly unpatentable over claims 6-9, 14-17 and 22-24 of copending application Serial No. 08/398,852.

The claims pending in the subject applications are drawn to distinct methods. The claims in Serial No. 08/398,852 are drawn to a method for treating peripheral diabetic neuropathy, and the new claims in this application (24-45) are drawn to a method for treating stroke or traumatic injury to the central nervous system. Therefore, this obviousness-type double patenting rejection should be withdrawn.

II. Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-4, 8-11, and 15-18 are rejected under 35 U.S.C. § 112, first paragraph, on the grounds that the specification is not enabling for effecting any changes in the biochemistry or function of the central nervous system. Applicant respectfully traverses this rejection.

The new claims added by the above amendment are drawn to treating stroke or traumatic injury to the central nervous system. The description and guidelines for carrying out these methods are disclosed in applicant's specification. For example, the specification discloses the

method as recited in the claims, IGF compounds and sources (p. 5, l. 20-p. 6, l. 9 and p. 7, l. 25-p. 8, l. 2), dosage ranges and adjustment parameters (p. 8, ll. 4-24); pharmaceutical composition variations (p. 8, l. 25-p. 9, l. 5); and parenteral administration techniques (p. 9, ll. 5-15). The Examples of the specification also demonstrate that IGF treatment can prevent and limit brain and spinal cord damage in rats. The rat model is reasonably predictive of the effectiveness of the claimed methods in other mammals, including humans.

Several research groups have conducted further experiments on rat and human models that parallel the disclosed and claimed methods. Those successful experiments further prove the operability of the methods disclosed and claimed in this application.

Saatman et al., "Insulin-like Growth Factor-1 (IGF-1) Improves Both Neurological Motor and Cognitive Outcome Following Experimental Brain Injury," *Exptl. Neurol.* 147:418-427 (1997), shows that subcutaneous IGF administration improves neurological motor and cognitive outcome in rat brain injury caused by percussion.

Fernandez, et al., "Insulin-like Growth Factor-I Restores Motor Coordination in a Rat Model of Cerebellar Ataxia," *Proc. Natl. Acad. Sci. USA* 95:1253-58 (1998), shows that subcutaneous IGF treatment can prevent neuron loss and loss of motor function in rats treated with a 3-acetylpyridine neurotoxin.

Hatton et al., "Intravenous Insulin-like Growth Factor-I (IGF-I) in Moderate-to-severe Head Injury: A Phase II Safety and Efficacy Trial," *J. Neurosurg.* 86:779-86 (1997), shows that IGF infusion into the circulation results in improved clinical neurological outcome scores in human patients suffering from traumatic brain injury in a phase II clinical trial.

"Pharmacological Treatment of Traumatic Brain Injury: Following in the Footsteps of Stroke," *Drug & Market Development* 9(3):60-64 (1998), shows that treatment of traumatic brain injury is predictive of stroke treatment. *Drug & Market* states that "The etiology of traumatic brain injury is closely linked to the etiology of stroke. Many of the pathological events that lead to severe disabilities and sometimes death are common to both. Most of the pharmaceutical agents used in treatment of traumatic brain injury were initially developed for stroke."

Loddick et al., "Displacement of Insulin-like Growth Factors from Their Binding Proteins as a Potential Treatment for Stroke," *Proc. Natl. Acad. Sci. USA* 95:1894-98 (1998), shows that increasing the IGF levels in the brain may be useful for the treatment of stroke and other neurodegenerative diseases. The experiments in Loddick et al. were conducted in a rat model acknowledged as being a clinically relevant model of stroke.

The Examiner appeared to indicate in the Advisory Action mailed January 16, 1998 (parent application Serial No. 571,802), that scientific articles published after the filing date are not proper evidence on the question of enablement. Applicant contends that those post-filing date articles are proper evidence of the operability of the methods disclosed in the application. The application as originally filed was adequate to enable one of ordinary skill in the art to make and use those claimed methods. The post-filing date evidence clearly demonstrates the operativeness of the methods in a mammal as taught and claimed in the patent application. That evidence cannot be disregarded by the examiner. Since they were published by third parties, the scientific articles also unquestionably provide unbiased evidence that applicant's teachings are operable.

Having shown that

(i) the specification tells one of ordinary skill in the art how to perform a method of treating stroke or traumatic injury to the central nervous system, and

(ii) researchers following those teachings have demonstrated operativeness of that method in rat and human models,

the enablement rejection should be withdrawn.

III. Rejection under 35 U.S.C. § 102(e)

Claims 1-6, 8-13, and 15-18 are rejected under 35 U.S.C. §102(e) as being anticipated by Lewis et al. Applicant respectfully traverses this rejection.

The Examiner states that "Applicant argues that the examiner has not established a case of enablement. However, the patent is presumed enabled by the examiner without evidence to the contrary."

Lewis simply does not mention an IGF molecule crossing the blood-brain barrier for the treatment of stroke or physical injury to the central nervous system. Lewis only refers to fusion proteins derived from IGF and another molecule. Those fusion proteins are not the subject of this application. Pages 5 and 6 of the specification even define IGF to exclude those fusion proteins: "For purposes of the invention, IGF-I does not, however, encompass fusion proteins of IGF-I and a non-IGF peptide" and "For purposes of the invention, IGF-II does not, however, encompass fusion proteins of IGF-I and a non-IGF peptide."

The Examiner has not shown that Lewis et al. even recognized the potential for treatments of the central nervous system with IGF-I or IGF-II across the blood-brain barrier. Instead, Lewis et al. teaches away from such a possibility by characterizing the blood-brain barrier as a problem:

> Where the polypeptide is intended for use as a therapeutic for disorders of the CNS, an additional problem must be addressed: overcoming the so-called "blood-brain barrier," the brain capillary wall structure that effectively screens out all but selected categories of molecules present in the blood, preventing their passage into the brain. While the blood-brain barrier may be effectively bypassed by direct infusion of the polypeptide into the brain, the search for a more practical method has focused on enhancing transport of the polypeptide of interest across the blood-brain barrier, such as by making the polypeptide more lipophilic, by conjugating the polypeptide of interest to a molecule which is naturally transported across the barrier, or by reducing the overall length of the polypeptide chain.

Lewis et al., col. 3, ll. 44-59. Lewis et al. then describes various means to get an IGF molecule across the blood-brain barrier, which means are limited to modifying the IGF molecule, *e.g.*, by chemical modification such as conjugation to cationized albumin (col. 6, ll. 50-55), substituting or deleting certain amino acids (col. 7, l. 52 - col. 8, l. 29), or by intracerebral injection (Example 4). Lewis et al. simply does not state that the IGF-I or IGF-II molecule alone can be an effective method for treating a disease or disorder of the central nervous system. As such, it cannot be a basis for rejecting applicant's claims defining such treatment of the central nervous system by parenteral nonintracranial administration of IGF-I or IGF-II.

With respect to the enablement issue, applicant has challenged the enabling character of the Lewis et al. reference. Applicant questions how Lewis et al. could enable an IGF molecule crossing the blood-brain barrier when it actually teaches away from crossing that barrier. That challenge overcomes the Examiner's "presumption of enablement."

Therefore, the rejection under 35 U.S.C. § 102(e) should be withdrawn.

IV. Conclusion

In view of the above Amendment and Remarks, Applicant believes that this case is in condition for allowance.

The Examiner is invited to contact the undersigned attorney at (713) 787-1686 with any comments relating to this application.

If any fees are required, including fees for additional claims, the Assistant Commissioner is authorized to deduct those fees from Arnold, White & Durkee Deposit Account No. 01-2508/CSUA019--1WAA.

Respectfully submitted,

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ABSTRACT

Mammals suffering from stroke or traumatic injury to the central nervous system can be treated by a method that involves parenteral nonintracranial administration to the mammal of an IGF-I or an IGF-II in an amount effective to treat the stroke or traumatic injury.

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Pharmacology

Displacement of insulin-like growth factors from their binding proteins as a potential treatment for stroke

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Contributed by Wylie W. Vale, December 5, 1997

ABSTRACT **Insulin-like growth factors I and II (IGF-I and IGF-II) play an important role in normal growth and brain development and protect brain cells from several forms of injury. The effects of IGFs are mediated by type-I and type-II receptors and modulated by potentially six specific binding proteins that form high-affinity complexes with IGFs in blood and cerebrospinal fluid (CSF) and under most circumstances inactivate them. Because brain injury is commonly associated with increases in IGFs and their associated binding proteins, we hypothesized that displacement of this large "pool" of endogenous IGF from the binding proteins would elevate "free" IGF levels to elicit neuroprotective effects c mparable to those produced by administration of exogenous IGF. A human IGF-I analog [(Leu^{24,59,60}, Ala^{31})hIGF-I] with high affinity to IGF-binding proteins (K_i = 0.3–3.9 nM) and n biol gical activity at the IGF receptors (K_i = >10,000 nM) increased the levels of "free, bioavailable" IGF-I in the CSF. Intracerebroventricular administration of this analog up to 1h after an ischemic insult to the rat brain had a potent neuroprotective action comparable to IGF-I. This novel strategy for increasing "free" IGF levels in the brain may be useful for the treatment of stroke and other neurodegenerative diseases.**

Insulin-like growth factors I and II (IGF-I and IGF-II) are multifunctional peptides essential for normal growth and development (1). Their biological actions are mediated by the type-I IGF receptor (2) and possibly the type-II IGF receptor, which is identical to the cation-independent mannose 6-phosphate receptor (3). In the circulation and interstitial fluids, including the cerebrospinal fluid (CSF), IGFs are almost entirely associated with one or more of at least six IGF-binding proteins (IGFBPs) that bind IGFs with high affinity, thus limiting their interaction with receptors, and potentially providing a "reservoir" of biologically inactive IGF (1). IGFs undoubtedly play an important role in brain development and may also be important after injury. IGF treatment protects the developing or adult brain from hypoxic-ischemic injury (4–7) and forebrain ischemia (8), induces myelination (9–11), and reduces neuronal death *in vitro* caused by diverse forms of injury (12–16). Paradoxically, injury to the developing or adult brain is commonly associated with increases in brain IGFs as well as their associated binding proteins (4, 17–26). Consequently, even though IGFs are elevated, they may be complexed with their binding proteins and unavailable to provide neuroprotection. The IGF system, therefore, provides a rather unique opportunity for utilizing an endogenous neuroprotective factor. We hypothesized that displacement of the large "pool" of IGF from the IGFBPs in the brain would elevate "free" IGF levels, increasing receptor activation to elicit similar actions to administration of IGF-I itself.

In the present studies, we examined the role of brain IGFs and IGFBPs in neuroprotection by comparing the effects of hIGF-I with the selective, high-affinity IGFBP ligand inhibitor, [Leu^{24,59,60}, Ala^{31}]hIGF-I in *in vitro* studies of release of "free" bioactive IGF-I from rat cerebrospinal fluid and in *in vivo* studies to evaluate their neuroprotective effects in a rat model of focal ischemia. Data suggest that IGFBPs, by neutralizing IGFs, may serve to limit the actions of the peptides under both physiological and pathological conditions. Furthermore, the results demonstrating potent neuroprotective effects of the IGFBP ligand inhibitor comparable to IGF-I suggest that this strategy for increasing "free" IGF levels in the brain may be useful for the treatment of stroke and other neurodegenerative diseases.

MATERIALS AND METHODS

Synthesis and Purification of Peptides. hIGF-I and hIGF-II were obtained from Sigma. [Nle^{29}]hIGF-I and [Leu^{24,59,60}, Ala^{31}]hIGF-I were synthesized by a solid-phase peptide synthesis procedure as described previously (27) by using a t-butoxycarbonyl-Ala-(oxymethyl)-phenylacetamidomethyl (PAM) resin on a Beckman 990 peptide synthesizer. Derivatized amino acids and resin used in the synthesis were purchased from Bachem. After the last residue was coupled onto the growing peptide chain, the protected peptide resin was treated with the low–high hydrogen fluoride cleavage procedure (28) to remove the peptide from the resin anchor and deprotect the side-chain functional groups. The crude peptide was extracted with 5 M guanidine HCl in 0.1 M NH_4OAc, and the pH of the extract was maintained at 5 with HOAc. After filtering off the resin, the solution was diluted with 0.1 M NH_4OAc to 2 M guanidine HCl to a peptide concentration of ≈1 mg/ml. The peptide was cyclized by air oxidation by stirring at room temperature for 24 h while maintaining the pH at 8.4 with 10% concentrated NH_4OH. After oxidation, the pH was adjusted to 5 and the solution was dialyzed against 0.1 M acetic acid at room temperature to remove the guanidine salt. The recovered dialysate was lyophilized and the crude product was purified by gel filtration on Sephadex G-50F, followed by carboxymethyl cellulose cation-exchange chromatography and preparative HPLC on a KP-100 Gradient HPLC system with a Vydac C18 cartridge (Biotage, Charlottesville, VA). The purified product was verified by mass spectrometric analysis on a SCIEX/AP1 LC/MS system equipped with an ion-spray source (Perkin–Elmer).

Radioligand Binding Assay. Human IGFBP-1, BP-4, and BP-5 were expressed in the BaculoGold Expression System (PharMingen) in Sf9 insect cells and purified by affinity

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Abbreviations: IGF, insulin-like growth factor; IGFBP, IGF binding protein; CSF, cerebrospinal fluid.

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chromatography on a hIGF-I-coupled Affi-Gel 10 column, followed by reverse-phase HPLC. Human IGFBP-2 and BP-3 were isolated from outdated plasma as described previously (29). The binding assay was performed at room temperature in duplicate in 0.02% Nonidet P-40/PBS buffer, pH 7.2. Two hundred microliters of a 2.5 nM IGFBP solution (0.5 pmol) was added to a 12 × 75-mm glass test tube. The reaction was started by the addition of 100 μl buffer, hIGF-I, hIGF-II, or [Leu^{24,59,60}, Ala^{31}]hIGF-I solution, followed by 100 μl of [^{125}I]hIGF-I (30,000 cpm, specific activity ≈2,200 Ci/mmol; New England Nuclear). After incubation for 2 h, 100 μl of 20% BSA and 500 μl of 20% PEG-8000 in the PBS buffer were added and the mixture was vortexed and then centrifuged for 30 min at 3,000 rpm. The supernatant was carefully removed by suction and the pellet was counted in a γ-counter.

Radioligand and Western Blot Analysis of the IGF-Binding Proteins. Twenty microliters of rat CSF was fractionated by SDS/PAGE and blotted onto nitrocellulose paper, and the blot was then incubated with [^{125}I]hIGF-I and examined by autoradiography according to the procedure described previously (30). Western blot analysis of the IGFBPs was performed by electrophoresing 20 μl of rat CSF per lane on SDS/PAGE, followed by blotting of the gel onto nitrocellulose paper, according to the published procedure (30). The nitrocellulose paper was then cut into replicate strips, and one strip was incubated with IGFBP-2 antiserum (Upstate Biotechnology, Lake Placid, NY) whereas the other was incubated with IGFBP-5 antiserum raised in a rabbit with a synthetic peptide fragment as described previously (30). The stained bands were revealed by incubation with peroxidase-conjugated goat anti-rabbit IgG, followed by chemiluminescence detection with a commercial kit (Pierce).

Gel Filtration Analysis of Dissociated IGF-I from the IGF-I/IGF-Binding Protein Complex. Five hundred microliters of rat CSF was incubated with [^{125}I]hIGF-I at 37°C for 1 h to incorporate the radioiodinated peptide into the complex, and the incubated fluid was divided into 100-μl aliquots. To each aliquot was added buffer (control), IGF-I, or [Leu^{24,59,60}, Ala^{31}]hIGF-I, and the mixture was incubated for 1 h at 37°C, followed by storage on ice. For gel-filtration analysis, each aliquot was diluted with 400 μl 0.02% NaN_3/0.1% BSA/PBS buffer and the diluted sample was loaded onto a 1 × 50 cm Sephadex G-50F column; the column was developed with the same buffer at a flow rate of 0.5 ml/min at room temperature. The collected fractions were counted in a γ-counter.

Fibroblast Proliferation Assay. Biological activities of hIGF-I, [Nle^{59}]hIGF-I, and the IGFBP ligand inhibitor [Leu^{24,59,60}, Ala^{31}]hIGF-I were tested in a BALB/c 3T3 fibroblast assay (31). The ability of the peptides to induce proliferation was measured by counting the amount of [^{3}H]thymidine incorporated by the cells. Cells were aliquoted to 96-well microtiter plates (180 μl per well). After a 48-h incubation at 37°C and 5% CO_2, the plates were washed twice with 0.1% calf serum/DMEM and incubated for an additional 24 h. Twenty microliters of sample and 1 μCi [^{3}H]thymidine (New England Nuclear) were added to each well, and the plates were incubated for a further 24 h. After incubation, the medium was removed and the cells were fixed by the addition of 200 μl of a 25% acetic acid/75% ethanol solution per well. After removal of the fixing solution, the plates were washed three times with cold 10% trichloroacetic acid and the cells were lysed in 200 μl 0.2 M NaOH. The entire 200 μl of lysate solution was transferred into a scintillation vial; 2.5 ml of scintillation liquid was added and the vials were counted in a γ-counter.

Rat Middle Cerebral Artery Occlusion Model of Focal Ischemia. Male Sprague–Dawley rats (Charles River) were housed in a 12-h light/12-h dark cycle and allowed food and water *ad libitum*. The experiment protocol was approved by the Institutional Animal Care and Use Committee in accordance with National Institutes of Health guidelines. Rats weighing 160–190 g were anesthetized with isoflurane (4% induction, 2.2% maintenance) in O_2, and indwelling guide cannulae were stereotaxically implanted in the right, lateral ventricle [coordinates in mm: lateral (+1.5); anteroposterior (−0.8); dorsoventral (−3.0), relative to Bregma] to permit subsequent injections into the CSF. Ten days later, rats were reanesthetized with halothane (4% induction, 2–2.5% maintenance) in O_2, and focal cerebral ischemia was induced by permanent occlusion of the left, middle cerebral artery (MCAo) proximal to the lenticulostriate branch, by electrocoagulation. Throughout surgery and recovery from anesthesia, animals were maintained normothermic by means of a heated blanket. Peptides or vehicle (sterile water) were injected into the lateral ventricle (in a volume of 5 μl) over 2–3 min, either concurrent with or 1 h after MCAo. One day after MCAo, animals were killed and the brains were removed. Delineation of the lesion was determined on fresh 500-μm coronal brain sections incubated in 2% tri-phenyl-tetrazolium chloride (TTC; Sigma) by using an indirect approach thus "correcting" for any swelling. Lesion volume was calculated for each brain by integration of the areas of infarct in each section. These procedures are described in more detail elsewhere (32). Peptide-treated groups were compared with vehicle-treated animals by using Student's unpaired *t* test.

RESULTS AND DISCUSSION

Before this study, Bayne *et al.* (33) had reported that an hIGF-I analog, [Leu^{24,60}, Ala^{31}]hIGF-I, has a >1,200-fold loss in affinity to the type-I IGF receptor and little measurable affinity for the type-II receptor. To facilitate the synthesis and stability of this analog, the endogenous methionine at position 59 was replaced with leucine. The resulting compound, [Leu^{24,59,60}, Ala^{31}]hIGF-I, was tested with hIGF-I and hIGF-II for displacement of [^{125}I]hIGF-I binding to human IGFBP-1, -2, -3, -4, and -5, and biological activity in BALB/c 3T3 fibroblast cells, which proliferate in response to IGFs. As shown in Table 1, hIGF-I and hIGF-II have comparable or somewhat higher affinities for IGFBP-1, -2, -3, -4, and -5 (K_i values = 0.01–0.22 nM) than for their homologous type-I and type-II receptors (K_i values = 1.5 and 0.2 nM, respectively). In contrast to the relative lack of selectivity of IGF-I and IGF-II between the IGFBPs and IGF receptors, [Leu^{24,59,60}, Ala^{31}]hIGF-I has high affinity for IGFBP-1, -2, -3, -4, and -5 (K_i values = 0.28–3.91 nM) and is inactive at IGF receptors (K_i values = >10,000 nM) (Table 1). Furthermore, in contrast to hIGF-I, which dose-dependently stimulated

Table 1 Relative affinity and selectivity of IGF-I, IGF-II, and IGFBP ligand inhibitor (IGFBP-LI) for IGF-binding proteins and the type-I and type-II IGF receptors

Protein or receptor	K_i, nM		
	IGF-I	IGF-II	IGFBP-LI
IGFBP-1	0.12 ± 0.03	0.051 ± 0.008	1.91 ± 0.10
IGFBP-2	0.06 ± 0.01	0.010 ± 0.005	1.92 ± 0.80
IGFBP-3	0.21 ± 0.04	0.023 ± 0.005	1.80 ± 0.20
IGFBP-4	0.10 ± 0.03	0.032 ± 0.004	0.28 ± 0.10
IGFBP-5	0.22 ± 0.04	0.040 ± 0.004	3.91 ± 2.40
Type-I receptor	1.5	3.0	>10,000
Type-II receptor	400	0.2	>10,000

The relative affinities of hIGF-I, hIGF-II, and the IGFBP-LI, [Leu^{24,59,60}, Ala^{31}]hIGF-I for the various IGFBPs were determined in radioligand-binding assays as described in *Materials and Methods*. Data represent the mean ± SEM of three separate determinations. The affinity constants of the IGF-I and IGF-II for the type-I and type-II receptors were taken from ref. 34 and 35, respectively, whereas the affinity constants of the IGFBP ligand inhibitor for the IGF receptors were taken from ref. 33.

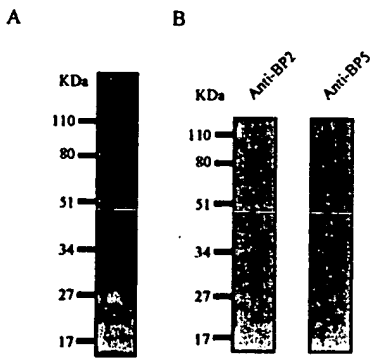

FIG. 1. Identification of IGF-binding proteins in rat cerebrospinal fluid. (*A*) Radioligand blot of IGF-binding proteins present in rat cerebrospinal fluid. Note that the major radiolabeled band detected has a molecular mass of ≈32 kDa, which corresponds to the molecular mass of IGFBP-2 or IGFBP-5. (*B*) Western blot identifying the major IGFBP in rat CSF as BP-2.

DNA synthesis in 3T3 fibroblasts with an IC_{50} of 5–10 nM, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I had no activity in the assay at concentrations of up to 8 μM, indicating a lack of interaction with the IGF receptors in this functional assay.

We evaluated the ability of the IGFBP ligand inhibitor, [$Leu^{24,59,60}$, Ala^{31}]hIGF-I, to displace the bound IGF-I and elevate "free" biologically active levels of the peptide in rat CSF and in the BALB/c 3T3 fibroblast proliferation assays. In agreement with previous reports (36, 37), using ligand and Western blot analyses, we determined that the most abundant IGFBP in rat CSF is BP-2 (Fig. 1 *A* and *B*, respectively). Gel filtration analysis of rat CSF that had been preincubated with trace quantities of [^{125}I]hIGF-I demonstrated that ≈64% of [^{125}I]hIGF-I eluted as a higher molecular mass complex (presumably bound to IGFBP-2) and ≈36% eluted at a molecular mass corresponding to "free" [^{125}I]hIGF-I (Fig. 2). Incubation of the [^{125}I]hIGF-I-incorporated CSF with either IGF-I (0.1 μM) or [$Leu^{24,59,60}$, Ala^{31}]hIGF-I (1 μM) resulted in a decrease in the proportion of IGF-I/IGFBP complex and a corresponding increase in "free" [^{125}I]hIGF-I levels (Fig. 2). The higher concentration of the IGFBP ligand inhibitor than IGF-I required to increase "free" IGF-I levels is in keeping with the ≈10- to 20-fold lower affinity of the IGFBP ligand inhibitor for IGFBPs than IGF-I itself (Table 1). The ability of the IGFBP ligand inhibitor to release bioactive IGF-I was further evaluated in the 3T3 fibroblast assay. Human IGF-I (3 nM) produced robust proliferation of 3T3 fibroblasts as reflected by increased [^{3}H]thymidine incorporation; the hIGF-I-induced proliferation was substantially blocked by addition of 20 nM IGFBP-2 (Fig. 3). The addition of [$Leu^{24,59,60}$, Ala^{31}]hIGF-I dose-dependently reversed the neutralizing effect of IGFBP-2 on IGF-I (ED_{50} = 200 nM), demonstrating the ability of the IGFBP ligand inhibitor to displace hIGF-I bound to IGFBP-2 (Fig. 3). Overall, these *in vitro* data clearly demonstrate that the IGFBP ligand inhibitor is capable of interacting with the binding protein in a specific manner to displace complexed IGF-I and release "free" bioactive peptide.

In view of the potent neuroprotective and regenerative effects of IGFs (4–16), we tested the hypothesis that displacement of IGF from its BPs in the brain could confer neuroprotection in a clinically relevant model of stroke in the rat. Although the models of ischemia [hypoxic-ischemia (4–7) and forebrain ischemia (8)] previously used to evaluate the effects of IGF-I provide important information on the effects of ischemia on the brain, they are not considered as models of stroke. It is generally accepted that occlusion of a single intracranial artery (the middle cerebral artery, MCA) provides the best model to study stroke (38). Because of the relatively large quantities of IGF-I required for *in vivo* studies, synthetic [Nle^{59}]hIGF-I was employed. In [Nle^{59}]hIGF-I the endogenous methionine at position 59 is replaced by the isosteric norleucine to eliminate the possibility of oxidizing the methionine to methionine sulfoxide during cyclization of the three disulfide bonds by air oxidation in the synthesis of the molecule. Bioassay of [Nle^{59}]hIGF-I and hIGF-I in BALB/c 3T3 fibroblasts showed no difference in the proliferative potency between the two compounds. Adult male rats previously implanted with lateral cerebral ventricular guide cannula were subjected to ischemia by permanent occlusion of the MCA (MCAo), and the resulting brain lesion was visualized and quantified 24 h later. Animals that had received a single intracerebroventricular (i.c.v.) injection of synthetic

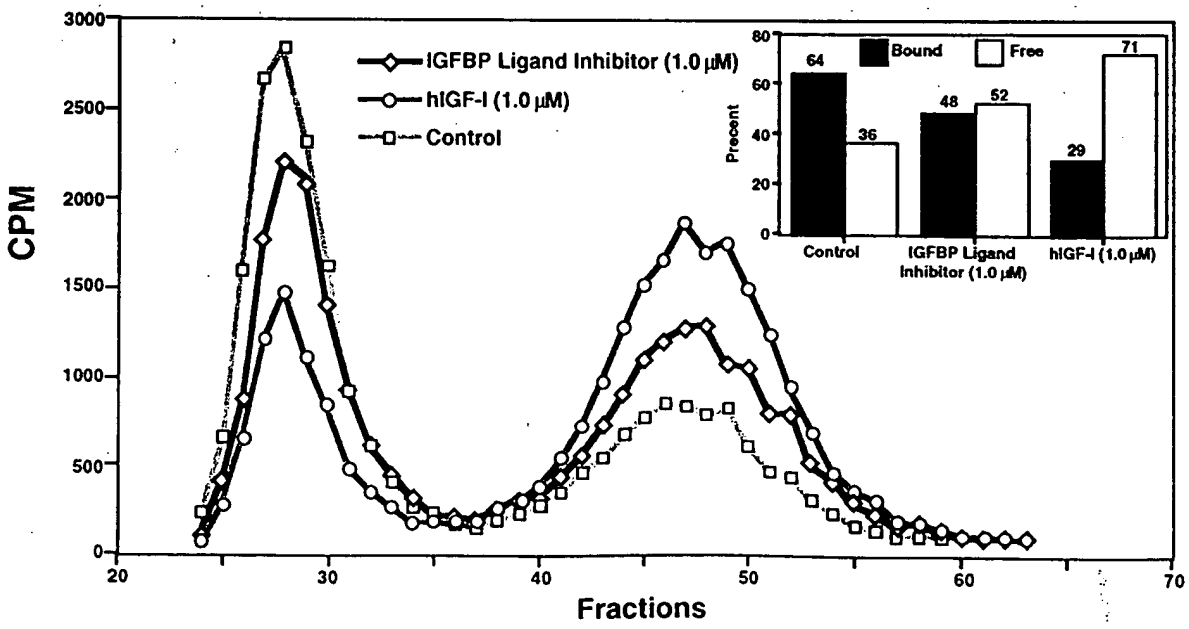

FIG. 2. Gel-filtration analysis of rat CSF showing the relative proportions of bound and "free" [^{125}I]hIGF-I dissociated by IGF-I or the IGFBP ligand inhibitor. The Sephadex G-50F gel-filtration profiles of rat CSF in the absence (Control) or presence of hIGF-I [hIGF-I (0.1 μM)] or 1.0 μM of [$Leu^{24,59,60}$, Ala^{31}]hIGF-I [IGFBP Ligand Inhibitor (1.0 μM)] are shown. The quantified data representing the relative proportions of the hIGF-I/IGFBP complex (Bound) and "free" hIGF-I (Free) are presented in the *Inset*.

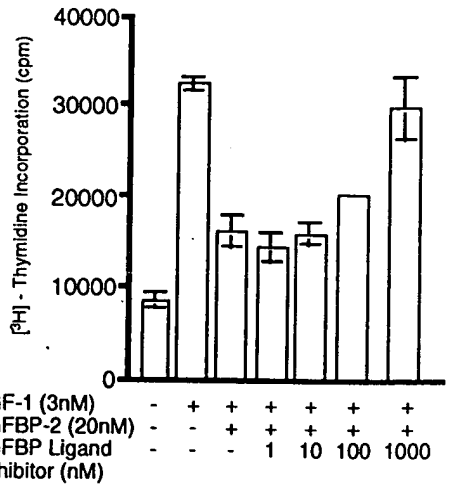

FIG. 3. Reversal of IGFBP-2 inhibition of hIGF-I-stimulated fibroblast proliferation by [Leu^{24,59,60}, Ala^{31}]hIGF-I *in vitro*. Human IGF-I dose-dependently stimulated DNA synthesis with an ED_{50} of 5–10 nM. In contrast, [Leu^{24,59,60}, Ala^{31}]hIGF-I did not induce DNA synthesis in 3T3 cells at any of the doses tested (0.1–8,000 nM). IGFBP-2 (20 nM) substantially inhibited the proliferative effect of 3 nM hIGF-I. Addition of IGFBP ligand inhibitor dose-dependently reversed this inhibition with an ED_{50} of 200 nM.

[Nle^{59}]hIGF-I (50 μg) or the IGFBP ligand inhibitor (50 μg) at the time of MCAo had much smaller total lesion volumes than those injected with vehicle, primarily because of a reduction of the cerebral cortical infarct volume, although some protection was also evident in the striatum (Fig. 4*A*). The extent of neuroprotection (40–50%) was comparable for [Nle^{59}]hIGF-I and the IGFBP ligand inhibitor and is in keeping with that seen after treatment with NMDA receptor antagonists (39, 40). Remarkably, the extent of protection was similar whether [Nle^{59}]hIGF-I or [Leu^{24,59,60}, Ala^{31}]hIGF-I was administered concurrent with (0 h) or 1 h after occlusion of the artery (Fig. 4*B*), providing a therapeutic window for the treatment as is available in this rat model of ischemia. In the more slowly developing hypoxic-ischemia model, IGFs confer neuroprotective effects when administered up to 2 h after the insult (4–7). These observations taken together with previous data indicating that i.c.v. injection of 50 μg hIGF-I has no impact on plasma glucose levels or body temperature of ischemic rats (7) suggest that IGFs protect neurons by interfering with the pathological pathways that are initiated after ischemia.

The mechanisms through which the IGFBP ligand inhibitor and IGFs produce their neuroprotective effects are at present unclear. Ischemic neuronal damage has been attributed, in part, to the extracellular accumulation of excitatory amino acids; in preliminary studies done in our laboratory, the IGFBP ligand inhibitor [Leu^{24,59,60}, Ala^{31}]hIGF-I attenuated the loss of pyramidal neurons in the hippocampus after intrahippocampal administration of quinolinic acid. In addition to producing their neuroprotective effects by interfering with endogenous mediators of ischemia such as glutamate, IGFs have the distinct advantage of also having the ability to act as regenerative growth factors. The IGFBP ligand inhibitor is capable of increasing the release of not only IGF-I but also IGF-II, which also has neuroprotective effects (13). The release of IGF-II in addition to IGF-I by the IGFBP ligand inhibitor may represent a therapeutic advantage over IGF-I treatment whose selectivity may be limited to actions at the type-I IGF receptor. Because neurodegeneration may be associated with lower levels of "free" bioactive IGFs, in part, because of increased brain expression of IGFBPs (4, 17–26), displacement of this "pool" of endogenous IGFs from their binding proteins with ligand inhibitors seems appropriate. The increased expression of brain IGFBPs (4, 17–26) may also serve to limit the actions of exogenously administered IGFs and provides strong support for the therapeutic relevance of IGFBP ligand inhibitors for the treatment of neurodegeneration. In addition, because the IGFBP ligand inhibitor approach achieves its effect by elevating local endogenous levels of "free" IGFs, a ceiling effect is reached when all the IGFs are released from IGFBPs, thus limiting the side effects that may occur after global activation of IGF receptors by exogenously administered IGF-I. This advantage is evident in other systems such as the corticotropin-releasing factor (CRF) family of peptides, in which the actions of the endogenous peptide(s) are limited by a CRF-binding protein (41). For example, CRF-binding protein ligand inhibitors, like CRF-receptor agonists, enhance learning and memory (42, 43) and blunt excessive weight gain (44) in a variety of rodent models. However, in

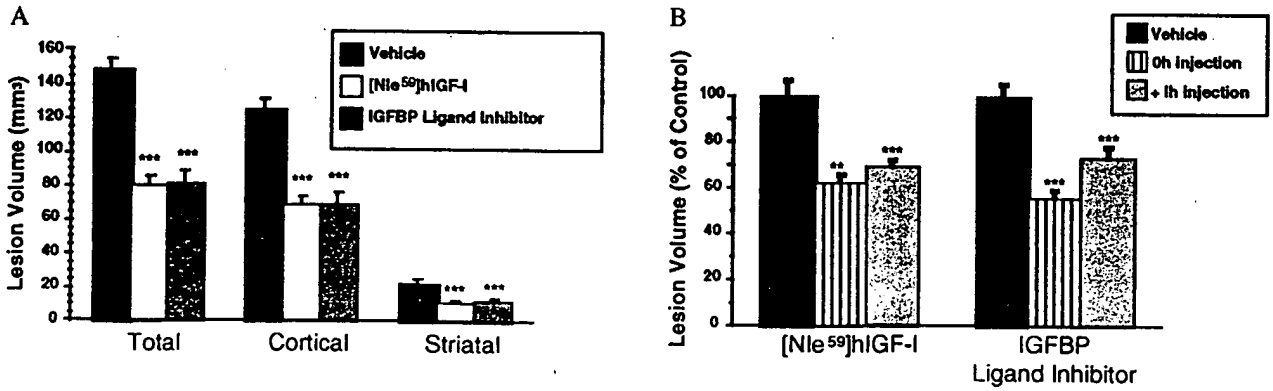

FIG. 4. The protective effects of [Nle^{59}]hIGF-I and the IGFBP ligand inhibitor on ischemic brain damage. (*A*) In the first series of experiments, the effect of concurrent administration of [Nle^{59}]hIGF-I or [Leu^{24,59,60}, Ala^{31}]hIGF-I was determined. Data are presented as mean lesion volume ± SEM. Animals injected at the time of MCAo with [Nle^{59}]hIGF-I (50 μg, n = 7, open bar) or the IGFBP ligand inhibitor, [Leu^{24,59,60}, Ala^{31}]hIGF-I (50 μg, n = 6, gray bar) had dramatically and statistically reduced lesion volumes compared with animals injected with vehicle (n = 6, solid bar). Protection was observed in cerebral cortical and striatal tissue. ***, $P < 0.001$. (*B*) The effects of delaying administration of [Nle^{59}]hIGF-I or the IGFBP ligand inhibitor [Leu^{24,59,60}, Ala^{31}]hIGF-I were determined in a separate series of experiments. Data are presented as the percentage of the mean lesion size of the respective vehicle-treated group (mean ± SEM). As observed in the previous experiment, animals injected at the time of MCAo with [Nle^{59}]hIGF-I (50 μg, n = 7, striped bar) or the IGFBP ligand inhibitor (50 μg, n = 6, striped bar) had dramatically and statistically reduced lesion volumes compared with animals injected with vehicle (n = 6, solid bar). When administration of the peptide was delayed to 1 h after MCAo, protection with [Nle^{59}]hIGF-I (50 μg, n = 7, gray bar) was remarkably similar, and protection with IGFBP ligand inhibitor (50 μg, n = 8, gray bar) was only slightly less than observed with concurrent administration. **, $P < 0.01$; ***, $P < 0.001$.

marked contrast to the effects of a CRF-receptor agonist, CRF-binding protein ligand inhibitors do not induce anxiety (42), stimulate adrenocorticotropic hormone secretion, or elevate heart rate and blood pressure (44). A further advantage in targeting IGFBPs is that it may be possible to identify nonpeptide small molecules that act as IGFBP ligand inhibitors, with the potential for good blood–brain barrier penetration and oral activity.

In summary, our data demonstrate that pharmacological elevation of "free" endogenous IGFs in the brain confers protection in a clinically relevant model of stroke. Because of the dramatic protection observed with this strategy, even when treatment is delayed for 1 h after occlusion of the artery, these data suggest that displacement of IGFs from IGFBPs in the brain is a potential treatment for stroke. Moreover, in view of the potent actions of IGFs on survival of neurons and glial cells as well as the widespread protective effects against a variety of brain insults, IGFBP ligand inhibitors may have broader utility for the treatment of various neurodegenerative disorders as well as traumatic brain and spinal cord injury.

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Table 8
Miscellaneous Drugs in Devel pment for RA

Drug	Status	Company	Comments
Therafectin™ (amiprilose)	Phase III	Boston Life Sciences, Inc.	Based on Phase III results, an NDA to be submitted for this synthetic carbohydrate inhibits IL-1, IL-2, PGE_2 and LTB_4 production
Reumacon™ (CPH 82)	Phase III	Conpharm AB	Orally available semi-synthetic derivative of two lignan glucosides
Colloral™ (AI-200)	Phase II	Autoimmune, Inc.	Solubilized type II chicken collagen for development of oral tolerance
ISIS-2302	Phase II	Isis Pharmaceuticals, Inc. (with Boehringer Ingelheim GmbH)	Phosphorothioate antisense drug inhibiting ICAM-1 expression that is also in Phase III for Crohn's disease
CGP-25019C	Phase II	Novartis	LTB4 antagonist also indicated for other inflammatory diseases
TA-650	Phase II	Tanabe Seiyaku Co., Ltd.	Undisclosed mechanism
CI-1004	Phase II	Warner-Lambert Co.	
RA tetrapeptide	Phase I	Peptide Therapeutics Group plc (with BTG plc)	For intra-articular injection to inhibit IgA-α1-antitrypsin complex
Verteporphin™ (BPD-MA)	Phase I	QLT PhotoTherapeutics, Inc. (with Beaufour-Ipsen Group)	Benzoporphyrin derivative that is light activated
Taxol™ (paclitaxel)	Preclinical	Angiotech Pharmaceuticals, Inc.	Anti-cancer drug (launched) with anti-inflammatory effects, including inhibition of neutrophils, synoviocyte proliferation, MMP production, and angiogenesis
Phosphosugars	Preclinical	ANUTECH Pty Ltd.	
Colloral™ (AI-200)	Preclinical	Autoimmune, Inc.	Recombinant human type II collagen for development of oral tolerance
Troponin I	Preclinical	Boston Life Sciences, Inc.	
Integrin adhesion inhibitor(s)	Preclinical	Cytel Corp. (with Sumitomo Pharmaceutical Co., Ltd.)	Small molecule inhibitors of VLA-4-mediated cell adhesion
Gene therapy	Preclinical	GeneMedicine, Inc.	
ARAs	Preclinical	Gensia Sicor, Inc.	
Bispan®	Preclinical	Inflazyme Pharmaceuticals, Ltd.	Unknown mechanism, derivative of contignasterol, a natural steroid
ISIS 3521	Preclinical	Isis Pharmaceuticals, Inc.	Antisense drug also in Phase I for cancer
Glucocorticoid agonist(s)	Preclinical	Ligand Pharmaceuticals, Inc. (with Abbott Laboratories)	
SMART® Antibodies	Preclinical	Protein Design Labs, Inc.	Humanized MAb directed against γ-IFN, CD3, or E- and P-selectin indicated for autoimmune diseases
Cyclic polyamine analogs	Preclinical	SunPharm Corp.	Polyamine analogs interfere with DNA replication, RNA transcription, processing and translation
TBC 772	Preclinical	Texas Biotechnology, Inc.	Cyclic peptide inhibitor of VCAM-1/VLA-4 mediated cell adhesion
HP-466	Preclinical	Trega Biosciences, Inc.	Melanocortin receptor agonist
Unnamed	Preclinical	Xenova Group plc (with Suntory)	Natural product screening

Source: D&MD

Abbreviations used: ARAs, adenosine regulating agents; ICAM, intracellular adhesion molecule; IFN, interferon; LTB4, leukotriene B4; VCAM, vascular cell adhesion molecule; VLA, very late antigen

Summary

RA is a disease target that presents a major market opportunity due to dissatisfaction with current therapies and the enormous patient population. Recent advances in basic research have uncovered numerous potential drug targets that appear ripe for exploitation. Since RA falls into the category of autoimmune diseases, it will be difficult to design drugs that are highly selective for mechanisms that are unique to RA pathogenesis. Many immunosuppressants, for example, may also compromise the body's ability to defend itself against infection and cancer. Likewise, the newly discovered signal transduction pathways may not be unique to cells involved in RA, thus inhibition of their activity may generate unexpected adverse events. Nevertheless, there are many new drugs in the pipeline that are potentially safer and more efficacious than existing therapies. For RA patients, this is clearly good news.

NERVOUS SYSTEM

Pharmacological Treatment of Traumatic Brain Injury: Following in the Footsteps of Stroke

- The devastating impact of traumatic brain injury in the American population results in severe lifelong disabilities and high healthcare costs. Traumatic brain injury is one of the leading causes of death in younger age groups and results primarily from accidents which occur as a result of participation in activities deeply established in the American lifestyle.
- The etiology of traumatic brain injury is closely linked to the etiology of stroke. Many of the pathological events that lead to severe disabilities and sometimes death are common to both. Most of the pharmaceutical agents used in

treatment of traumatic brain injury were initially developed for stroke. Clinical trials for traumatic brain injury have followed this same pattern.

- The incidence of traumatic brain injury is increasing slightly every year while the incidence of stroke is decreasing. The need for effective pharmacological therapy is at its pinnacle.
- This article was prepared by Keith Baker, Brain Cede, Inc.

Overview

Traumatic brain injury (TBI) is an insult to the brain, typically from an object striking the head in incidents such as car accidents, falls, firearms associated with assaults, and sports/recreation activities. The initial event associated with TBI is damage to the brain caused by a traumatic blow to the head, often without penetration of the skull (i.e. closed head injury). The result of this initial trauma leads to severe subsequent injury which evolves from minutes to hours to days after the initial trauma. The outcome of serious insults to the brain include impairment of the cognitive, physical and psychological skills necessary for everyday living, and in many cases can lead to death. Five hundred thousand people are admitted to the hospital each year for TBI, of which approximately one-half will die within the first two hours of hospitalization. As a result of severe head injury, many of those who survive will sustain lifelong disability requiring rehabilitation services to live a normal life. The total impact of TBI in the US alone depicts some jolting statistics regarding the population afflicted, economic impact, morbidity and mortality (see Table 9).

Over the past 20 years, injury has become the leading cause of death in people under the age of 45. The majority of these injuries and resulting mortality are due to TBI. Unlike other neurological disorders such as stroke and the neurodegenerative diseases, where the majority of people afflicted are age 65 and over, the morbidity and mortality in the TBI population of the US is comprised of a much younger and gender-biased populace. The highest incidence of TBI victims is in males 14 - 25 years of age, followed by children and the elderly. Furthermore, males are twice as likely as females to sustain TBI due to lifestyle and the frequency of high-risk exposure. Brain injuries, in general, kill more Americans under the age of 34 than all other diseases combined.

Table 9
Impact of TBI in the US Annually

Scope	500,000 new cases; 1,000,000 existing
	95,000 lifelong disabilities
Mortality	75-100,000
Age Prevalence	Males age 14 to 24 years
Costs	Total costs: $48.3 billion
	Hospitalization and care: $31.7 billion
	Fatality Costs: $16.6 billion
	Individual: $4.0 million
Causes	Car Accidents: 50%
	Falls: 21%
	Firearms (assaults and violence): 12%
	Sports/recreation: 10%
	Other: 7%

Sources: Trauma Data Coma Bank, American Brain Injury Association

Table 10
Elements of Brain Injury

Primary Injury	Secondary Injury
Skull fracture	Brain swelling/edema
Contusion (bruises) at the site of physical impact	High intracranial pressure
Hematoma (blood clots due to damaged blood vessels)	Cerebral ischemia
Lacerations (tearing of tissue or blood vessels)	Intacranial infection
Diffuse axonal injury (injury to the connecting fibers of the nerve cells)	Coma
	Diffuse axonal injury

Source: American Brain Injury Association

Causes

The leading causes of TBI are random accidents which occur as part of everyday living. A random blow to the head can set off a variable and complex pathologic process in the brain. In the case of TBI, the brain may be injured in a specific location or the injury may be diffused to many different areas of the brain. The extent of the primary mechanical lesion can be amplified by secondary and delayed injury processes leading to ischemia and subsequent neuronal degeneration in brain regions away from the site of the lesion center. The path of brain injury leading to loss of brain cells is a continuous course of events, which is divided into primary and secondary injury for means of simplification (see Table 10).

Current acute clinical efforts for improving the outcome of the severely head injured patient center around treatment of the secondary events alone. This approach is based on the belief that the primary insult is immediate and irreversible. However, one must remember that the severity of the overall injury and the ability to provide treatment is determined during the first few moments of the primary injury. Many of the primary injuries remain throughout the secondary period, thus requiring treatment along with the secondary events. Contusions, hematoma and lacerations of the brain, singularly or together, may well be the genesis of the life-threatening secondary events.

Delayed secondary injury at the cellular level has come to be recognized as a major contributor to the tissue loss following TBI as well as the ultimate status of a patient's outcome. A cascade of pathologic events involving physiological, vascular and biochemical alterations begins immediately in response to the primary injury. The path to cell death involves changes in neuropeptides, ionic homeostasis, cellular and vascular permeability, neurotransmission, cerebral blood flow and cerebrospinal fluid flow. The severity of secondary injury is at the center of most of the disabling consequences of TBI. The success of therapy aimed at the amelioration of secondary events is the deciding factor upon which the clinical outcome of patients is based.

Evaluation

The level of consciousness (coma level) is an important indicator of the overall brain injury, as well as a sufficient predictor of patient outcome. While a patient is in a coma, a

Table 11
The Glasgow Coma Scale

Patients are scored for each of the three areas below.
Categorization of the status of brain injury using the GCS score is as follows:
Severe:3-8, Moderate:9-12, Mild:13-15

Motor response	6) Obeys commands; 5) localizes to pain; 4) withdraws to pain; 3) abnormal flexion of limbs; 2) abnormal extension of limbs; 1) no movement
Verbal response	5) Oriented and comprehensive; 4) confused conversation; 3) inappropriate; 2) incomprehensible sounds; 1) no sound
Eye opening	4) Spontaneous; 3) to speech; 2) to pain; 1) no eye opening

Source: Traumatic Brain Injury Association

variety of neurological evaluations can be performed. Various coma scales which represent the general responsiveness to sensory stimulation are a critical part of evaluation. One of the more widely used scales, the Glasgow Coma Scale (GCS), is usually administered upon admission to the hospital (see Table 11). The coma evaluation includes a neurological examination followed by tests of verbal and painful stimuli, in addition to simple observation of the patient over time. Other diagnostic tools include electroen-cephalograms (EEGs) and evoked potentials (EP) used to monitor neurophysiologic status. Measurements of cerebral blood flow and intracranial pressure are used to evaluate the level of coma by determining the amount of oxygenated blood going to the brain. The computed tomography (CT) scan is used to diagnose skull fractures, leaking of blood into the brain cavity which could cause a hematoma, and the effects of compression on the brain from these events. Narrowing of cerebral arteries in the brain, or vasospasm, is detected using unique methods of CT to scan cerebral vessels injected with radioactive chemicals.

Treatment

Treatment protocols vary according to the type of accident, early GCS scores, early evaluation criteria and location of the treatment center. Large and small emergency centers have diverse methods for the acute care of TBI victims. However, despite this variability, there are certain standardized procedures that are routinely followed in cases of TBI across the US. For example, increased intracranial pressure (ICP) is a major pathological factor for which proper treatment must be given. Due to excessive water in the brain (edema), ICP results in compression of cerebral tissue and eventually neuronal death. Universally, the method of treatment for ICP is hyperventilation, elevation of the head, cerebrospinal fluid removal (shunting) and medication. Surgical intervention is employed for the evacuation of hematoma and decompression of skull fractures. Seizures are common sequelae of TBI throughout the course of hospitalization and are treated with conventional anti-seizure medication. Due to the lack of any one effective therapeutic agent, no common pharmacological therapy is routinely employed in all centers for TBI.

Overview of Drug Therapy in TBI

Historically, therapeutic intervention in TBI, and most neurological disorders for that matter, has been limited by a lack of (1) understanding of the cause of the disorder, (2) reliable animal models which mimic the human disorder to test product candidates, and (3) adequate concentrations of drug reaching the target site, in this case the brain. Over the past ten years, the biomedical community (both academia and industry) has expanded its investment into fundamental research to allow significant gains in the understanding of neurological disorders, with the goal of developing successful therapeutic agents. With the advent of new screening tools for small molecules, discovery of neurotrophic factors, and gene therapy/cell-based drug development strategies, scientists are offering novel pharmacological approaches to the treatment of a variety of neurological disorders . In particular, concentrated research efforts in stroke, TBI's etiological sibling, have resulted in an overwhelming amount of knowledge regarding both disorders. The focus on stroke as the third leading cause of death in the US has provided a similar interest in the discovery of the pathophysiological events and the possible routes of pharmaceutical intervention associated with TBI. Other than corticosteroids, few drugs have been used consistently, and very few drugs have been originally designed specifically for the TBI population over the past 30 years. In actuality, many of the pharmaceutical agents that have been implicated for stroke have also been used in preclinical investigations of TBI, with moderate clinical use (see Table 12).

Synergistic Mechanisms Between Stroke and TBI

Stroke, TBI and spinal cord injuries are classified as traumatic and acute neurological disorders characterized by rapid onset of debilitating events as a result of some insult to nervous tissue. Unlike a neurodegenerative disease that may develop over many years, these disorders have rapid, destructive processes that lead to more nerve cell damage than is caused by the original trauma. Arising from a brief, unanticipated event (trauma or blood clot), the predominant pathology results in depleting the brain of blood, oxygen and glucose by cutting off the blood supply to the core region of the brain—a circumstance known as cerebral ischemia. Cerebral ischemia is the underlying pathology of stroke. Cells in the core region will die regardless of interventional therapy, but cells on the periphery of the core region, termed the penumbra, are the target cells for drug therapy. Nerve cells in the penumbra are not injured as a result of the original trauma, but are at high risk of death due to a pathologic cascade of events following the initial event. The therapeutic goal in acute stroke and head trauma intervention has been aimed at salvaging the cells in the penumbra region and thus limiting the overall brain damage that may occur under these conditions.

Cerebral ischemia occurs in approximately 30-50% of the TBI patient population, and many of the pathophysiological events leading to cell death found in cerebral ischemia are found in TBI. Thus, it is not the actual primary injuries that begin the cascade of molecular events, but rather the existing lethal cascade itself that the two disorders have in common. Evidence to support this hypothesis is found in the overabundance of research compiled throughout the last 20 years investigating the mechanisms of neuronal cell death in

stroke and TBI. The search for new therapeutic agents along with the basic research defining the etiology of these disorders has helped to fortify the similarities between stroke and TBI. Past drug strategies have been to intervene in the metabolic, biochemical and neurotransmitting processes that exist between neurons on a path to death. The group of agents assuming these characteristics are termed neuroprotectants.

Neuronal Cell Death and Neuroprotection

Calcium channel blockers. Following injury to the brain, metabolism of cells is reduced resulting in depletion of energy. The energy needed to maintain the calcium (Ca) pump is no longer available, thus allowing excessive Ca influx into the cell. The resultant overload of intracellular Ca causes neuronal cell death through multiple mechanisms including activation of proteases, cytoskeletal disruption and membrane damage. The prevention of increased Ca influx into the cell has been addressed by a number of potent channel blockers. Cell membranes have at least nine known Ca channels for maintenance of the ionic and water balance in the cell. Nimodipine is a popular Ca channel blocker that has been used in both stroke and TBI patients.

Excitatory amino acid (EAA) antagonists. The acidic amino acids glutamate and aspartate are neurotransmitters in the brain. An overproduction of EAAs causes excitation or overstimulation of cells after injury to the brain. Cell death is mediated through overstimulation and Ca influx into the cell. The EAA agonist *N*-methyl-D-aspartate (NMDA) can be blocked at its specific receptor site with a variety of agents that act as antagonists. This antagonism results in a reduction of calcium influx and prevention of cell death. Ketamine, dextrorphan, magnesium and dizocilpime (MK 801) are some agents that act as NMDA antagonists. Efficacy in preclinical investigations of TBI and stroke, as well as other neurological impairments, have been demonstrated.

Free radical scavengers and antioxidants. After CNS injury, the accumulation of oxygen free radicals (highly reactive chemical species) are produced via a number of pathways. The superoxide ion, hydrogen peroxide and the hydroxyl radical are produced in sufficient amounts to induce damage to the cell walls of neurons and cerebrovasculature. Oxygen free radicals can be scavenged by compounds such as superoxide dismutase and catalase, or antioxidants such as allopurinol and dimethyl sulfoxide. Efficacy using these compounds has been reported in cerebral ischemia and TBI.

Glucocorticoids. Steroids are endogenous compounds that reduce edema and intracranial hypertension through various mechanisms involved in the inflammatory response. Recent evidence suggests that steroids may play a role in inhibition of lipid peroxidation, which is responsible for the breakdown of cell wall components through the production of peroxide radicals. The traditional steroids such as methyl prednisolone have conflicting results in their efficacy in stroke and TBI. A newer class of compounds that work as inhibitors of lipid peroxidation only, termed lazaroids, have been reported to be effective in cerebral ischemia, spinal cord injury and subarachnoid hemorrhage, with minimal evidence of efficacy shown in preclinical studies of TBI. Tirilizad and U74006 are examples of the lazaroids used in TBI.

Status of Drug Therapy in TBI

As one might expect, the course of pharmacological therapy for the TBI population remains, at least in part, a duplication of therapeutic intervention for stroke. The most recent TBI clinical trials incorporate many of the same classes of drugs previously used in clinical applications for both disorders (see Table 13). The current US market for a drug with improved patient outcomes for stroke is estimated to be $300 million. The potential patient population for TBI is more than half that of stroke. By simple extrapolation, one would estimate the TBI market in the US alone to be in the area of $150 million. Given the fact that the development costs are negligible when applied to both clinical settings, a significant financial gain could be attained.

The mechanisms through which a drug can intervene in TBI are many, and the search for new drugs is of high priority. Research has provided substantial evidence to indicate that the regulation of calcium and free radical production is important in the treatment of TBI. With this in mind, the current strategy for drug discovery is being streamlined and many new versions of the same classes of drugs are being investigated with an emphasis on Ca regulation and free radical production. The new drugs in these classes are more potent compounds incorporating highly specific active sites. New classes of drugs that intervene as neuroprotectants at different points of the pathologic cascade are also in development. Some drug targets include glutamate receptors, glycine NMDA receptors, opioids, neurotrophic factors, cytokines and sodium channels. The trend in TBI research is an approach to this affliction as a worthy target for research in its own right, not simply as a secondary consideration to stroke.

Table 12
Drugs That Have Been Investigated in Clinical Trials of Stroke and TBI

Therapeutic Agent	Mechanism of Action	Status
Citicoline	Prodrug; precursor to constituents of cell membrane and neurotransmitter	Marketed in Europe for stroke and TBI; in clinical development in the US by Interneuron Pharmaceuticals; demonstrated significant long-term improvement in GCS for TBI
Tirilizad mesylate	Oxygen free radical scavenger	Originally developed for stroke but showed no efficacy; reached Phase III for TBI but was suspended due to no significant improvement
Methyl prednisolone	Steroid; reduce cerebral swelling	Approved for stroke in the US; various contradicting reports in the clinic, some demonstrating efficacy, some no improvement for TBI
PEG-SOD	Oxygen free radical scavenger	Originally developed for stroke but showed no efficacy; completed Phase III for TBI but showed no significant improvement
SNX-111	Calcium channel antagonist	Phase II for stroke; Phase III ongoing for TBI, early analysis shows improvement in GCS, cerebral edema and diffuse axonal injury

Source: D&MD.

Table 13
Recent Clinical Trials for TBI in the US

Company	Therapeutic Agent	Mechanism of Action	Clinical Status	Efficacy
Cambridge Neuroscience	Aptiganel	NMDA antagonist	Phase III	Results due in early 1998
Pharmacia & Upjohn	Tirilizad mesylate	Oxygen free radical scavenger	Phase III-suspended due to no significant improvement	Two trials showed no consistent effect
Cortech	CP-0127	Bradykinin-2 receptor antagonist	Phase II-suspended due to adverse effects in preclinical investigation	Initial clinical improved Glasgow Coma Scale and intracranial pressure
Neurex	SNX-111	Calcium channel antagonist	Phase III	Early analysis showed improvement in GCS, cerebral edema and diffuse axonal injury
Cypros	CPC-211	Neuroprotective; reduces lactase	Phase II	Well-tolerated and reduced levels of lactic acid in brain

Source: D&MD

Future Directions for TBI Therapy

There is verifiable evidence that the etiology of the traumatic/ischemic disorders are the result of a complex series of events that contain more than one path to neuronal cell death. For example, the entry of Ca into the cell, production of free radicals, and the inflammatory process leading to edema are three known events that occur simultaneously during TBI and stroke. New research in experimental animal models has examined the use of more than one drug to intervene in these individual processes, with various reports of Ca channel blockers and antioxidants, NMDA antagonists and steroids, and other various combinations being administered at the same time. Another approach using *combination therapy* is to link two active sites on one compound that may be protective at more than one site along the cascade of pathogenic events (i.e. certain compounds having both SOD and catalase activity, which could scavenge the superoxide free radical and the peroxide radical, respectively). Positive preliminary results have been reported with both approaches in animal models of TBI and stroke.

It is of great importance to not only take advantage of the similarities between TBI and stroke, but to also acknowledge a critical difference in these two neurological disorders. Due to the nature of TBI, despite rigorous research into both disorders, the lack of progress made in decreasing the incidence of TBI when compared to stroke is stunning. Through constant efforts made by the medical community to increase education and public awareness, the incidence of stroke has decreased by more than 50 % over the past 20 years. In contrast, the incidence of TBI has increased slightly by 7-8% over the past 20 years. The fact remains that without successful intervention, TBI will most likely continue at a steady rate to be a major cause of debilitation and/or death in the US population. The ultimate solution to this problem is to effectively enforce preventative measures; however, based on statistics for TBI over the past 20 years, this does not seem likely in the near future. Thus, there is an urgent need for effective pharmacological intervention to prevent the life-threatening cascade of pathological events caused by TBI before they are irreversible.

TECHNOLOGY & STRATEGY

Genomics: A Brave New World for Therapeutics Development

- Over the past two decades, many new technologies have provided the rationale for scientific study, a platform for the formation of small, specialized biotechnology companies and the promise of new therapeutics. Genomics, while providing and promising the same, is unlike any other.
- Genomics is different for several reasons. First, it is an enabling technology and not a product technology in and of itself. Second, although some product opportunities may be suggested in the near term, the big payoff of genomics will only come after considerably more DNA sequence information is accumulated, new allied technologies such as bioinformatics mature, and more basic research related to the function and expression of genes is performed in an effort to design ways to apply acquired information to the process of therapeutic development. Third, genomics will ultimately have a major impact on virtually every area of therapeutics in existence today and may enable the development of new therapeutic approaches which we cannot currently envision.
- In short, genomics is a technology for the long run—a technology which no one involved in therapeutic development can ignore in the short run.
- This article briefly traces the development of genomics and highlights the current technologies upon which genomics is being developed. The intent is to place genomics within the broad context of modern therapeutics development, identify the major players in the field and summarize the trends and expectations which have been formed around the technology. Selected information for this article was derived from the comprehensive D&MD report (#938) entitled *Genomics and Human Therapeutics Development*.
- This article was prepared by James W Hawkins, Ph.D.

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Cover photograph: This issue contains a colloquium entitled "Neuroimaging and Human Brain Function" (pages 763–929). The cover displays a montage of data during a word generation task from new techniques of functional brain imaging. The upper left image shows positron emission tomography (PET) data, the bottom image shows a similar section from a functional magnetic resonance imaging (fMRI) study, and the upper right image is the same fMRI study projected onto a fully unfolded surface of a human cerebral hemisphere. Each image shows areas of increased brain activity in color. The line through the three images is an fMRI time response to individual word generation events obtained at the location of the brightest response in the bottom fMRI image. Figure courtesy of Randy Buckner and Anders Dale at the Mallinckrodt Institute of Radiology at Washington University, St. Louis, and the Massachusetts General Hospital Nuclear Magnetic Resonance Center, Boston.

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Neurobiology

Insulin-like growth factor I restores motor coordination in a rat model of cerebellar ataxia

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ABSTRACT **We tested the potential of insulin-like growth factor I (IGF-I) to induce functional recovery in an animal model of cerebellar ataxia because this motor impairment is accompanied in humans and rodents by distinct changes in several components of the IGF-I trophic system. Rats rendered ataxic by deafferentation of the cerebellar cortex with 3-acetylpyridine recovered motor function after IGF-I was administered, as determined by behavioral and electrophysiological tests. When treated with IGF-I, inferior olive neurons, the targets of the neurotoxin, were rescued to various degrees (from 92 to 27% of surviving neurons), depending on the time that treatment with IGF-I was initiated. Furthermore, full recovery was obtained regardless of the route by which the trophic factor was administered (intraventricular or subcutaneous) even in rats with severe neuronal loss. These results suggest that human ataxia could be treated with IGF-I by a simple procedure.**

Motor pathways in the cerebellum and brainstem express several components of the insulin-like growth factor I (IGF-I) trophic system and are modulated by IGF-I in a paracrine/autocrine fashion (1, 2). Available evidence indicates that this trophic peptide is involved not only in coordination of the development of the cerebellum but also in maintenance of its adult function (3). Moreover, recent observations indicate that neurodegeneration of cerebellar pathways is related to changes in the IGF-I system in humans and in rodents (4–6). A common feature in these cases of neurodegeneration is a depletion of IGF-I levels in both serum and brain tissue. In addition, changes in endogenous IGF-I input by diverse experimental approaches modulate the response of the cerebellum to deafferentation (7). These data strongly suggest that neuronal death in the cerebellum, and possibly in other areas also (8), is related to deficits of IGF-I neurotrophic input.

Although the use of neurotrophic factors for treatment of neurodegenerative illnesses and even brain aging is currently widely advocated (9), strong experimental evidence in favor of their use in humans is at best scarce and mainly based on *in vitro* observations and preliminary success in animal models (10). Hence, detailed studies in experimental models of human neurodegenerative diseases are very much needed. By using a well-characterized rat model of human cerebellar ataxia (11), we have done a detailed study of the role of the IGF-I trophic system in the pathogenesis of this disease. Because our observations indicated that inappropriate IGF-I input to the cerebellum could underlie the inability of these animals to recover motor coordination, we administered recombinant IGF-I to them. Prolonged infusion of IGF-I to ataxic rats resulted in full recovery of motor coordination, whereas vehicle-treated animals showed no recovery at all.

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MATERIALS AND METHODS

Experimental Design. We used rats injected with 3-acetylpyridine (3AP), a neurotoxin that selectively destroys inferior olive neurons in the brainstem (6), as an animal model of human olivo-cerebellar degeneration resulting in cerebellar ataxia. We determined the potential therapeutic effects of IGF-I on this model of ataxia by assessing diverse parameters of olivo-cerebellar function. These included behavioral, biochemical, electrophysiological, and morphological tests.

Rat Models of Ataxia. Induction of ataxia in adult rats was achieved either by intraperitoneal injection of 3AP (50–65 mg/kg of body weight) (11) or by bilateral electrocoagulation of the inferior olive [applied current of 10 μA for 1 sec, with a monopolar electrode; stereotaxic coordinates (12) were 10.5 mm ventral, −3.8 mm from lambda, and ±0.6 mm lateral]. Because both procedures destroy the inferior olive, the cerebellar cortex losses its climbing fiber input and as a result the rats become permanently ataxic. Thus, after 3AP injection rats showed clear motor deficits including staggering of their hind limbs and gait disturbances. In addition, the animals showed a 10-fold reduction in the time spent in the rota-rod as compared with intact animals (see Fig. 1). Ten animals were used for each experimental group.

Evaluation of Motor Coordination. Motor coordination of ataxic animals was determined by two different tests. In the first one, animals were placed in a rota-rod apparatus (Ugo Basile, Italy) with increasing acceleration. The apparatus consisted of a horizontal motor-driven rotating rod in which the animals were placed perpendicular to the long axis of the rod, with the head directed against the direction of rotation so that the rat has to progress forward to avoid falling. The trial was stopped when the animal fell down or after a maximum of 5 min. The time spent in the rotating rod was recorded for each animal and trial. Animals received a pretraining session to familiarize them with the procedure before being rendered ataxic. Thereafter, a total of six consecutive trials were done for every animal in each session. Only the results from the sixth trial session for each animal were used for statistical comparison (see Fig. 1*A*). Animals were tested every 5 days in the rota-rod after injection of 3AP. In a second type of test, we used the inclined plane paradigm that measures the animal's ability to maintain its body position for 5 sec on an inclined plane covered with a corrugated rubber mat (13). The angle of the surface is changed from 10° to 90° at 5° intervals until the animal is unable to remain in position. Animals were placed in three positions: head up, right side up, and left side up. The average score for these three positions was used for all data analysis. Animals were tested at day 30 of the study (last time point in Fig. 1).

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Abbreviations: IGF-I, insulin-like growth factor I; 3AP, 3-acetylpyridine; i.c.v., intracerebroventricular; GABA, γ-aminobutyric acid.
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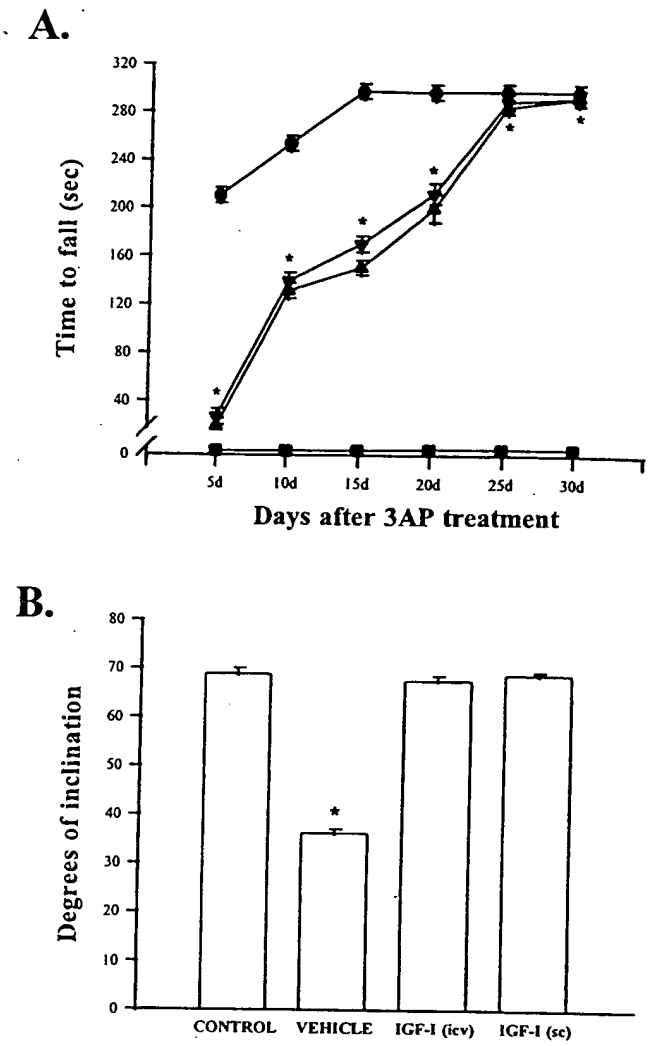

FIG. 1. Motor coordination in ataxic rats is normalized by IGF-I treatment. (*A*) Motor performance of 3AP-injected animals in the rota-rod test. Although untreated 3AP rats (vehicle group) were unable to stay in the rotating rod for longer than 2–4 sec, 3AP rats treated with IGF-I s.c. or i.c.v. showed a gradual recovery of their motoric abilities and reached normal performance (300 sec in the rotating rod) after 1 month of constant infusion. Statistical significance was determined by one-way analysis of variance followed by Student's *t* test in all experiments. *, $P < 0.001$ vs. vehicle-injected rats for both IGF-I-treated groups. ●, Controls; ■, 3AP + vehicle; ▼, 3AP + IGF-I (s.c.); ▲, 3AP + IGF-I (i.c.v.). Error bars in the vehicle group are smaller than the symbols. (*B*) Motor performance in the inclined plane task (13). The average score for the inclined plane task was 69.9 ± 1.3° (including all three orientations) in both control and IGF-I-treated 3AP animals. However, vehicle-treated 3AP rats exhibited a marked decrease in scores in the inclined plane. *, $P < 0.001$ vs. all other groups. Results shown in these and subsequent figures are mean ± SEM. Number of animals per group was 10. The two vehicle groups used (receiving 3AP plus either s.c. or i.c.v. osmotic minipumps filled with vehicle, $n = 10$ for each group) gave identical results and are pooled in a single group for clarity.

IGF-I Administration. IGF-I was administered to the rats either simultaneously with 3AP (50 mg/kg, i.p.) or 5 days after the injection of the neurotoxin. In both cases, animals showed clear symptoms of ataxia (see *Results*) well before any beneficial effect of the trophic factor became apparent. Prolonged IGF-I treatment was carried out with osmotic minipumps (Alzet 2002; infusion rate, 0.5 μl/h for 14 days). Pumps were filled with IGF-I by following the manufacturer's instructions. Minipumps were replaced with new ones after 2 weeks to maintain constant IGF-I infusion for an additional 2-week period. Attached cannulas were implanted stereotaxically in the lateral cerebral ventricle [coordinates (12): −0.8 mm anteroposterior, 4 mm ventral, and 1.5 mm lateral; IGF-I at 100 μg/ml). For subcutaneous delivery, pumps (filled with IGF-I at 1 mg/ml) were placed in the back of the animal in the scapula. Doses of IGF-I were chosen as described in published protocols (7). Control animals received pumps containing the saline vehicle. After 4 weeks of treatment with IGF-I, animals showed a minor, but not significant, increase in blood glucose levels: 79.5–82.1 mg/dl in controls and 84.6–87.6 mg/dl in IGF-I-treated 3AP animals ($n = 10$ per group).

Electrophysiology. Parasagittal slices (350 μm thick) from the cerebellar vermis were obtained from the different groups of rats by using a vibroslicer. Slices were incubated for 1 h in oxygenated buffer solution containing 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $MgSO_4$, 2 mM $CaCl_2$, 26 mM $NaHCO_3$, and 10 mM dextrose. Recordings were made in a submerged chamber with the slices perfused with oxygenated buffer solution (flow, 2–3 ml/min) at 30°C. Extracellular recordings were made with a glass micropipette filled with 1 M KCl or buffer solution. A bipolar stimulating electrode (FHC, Bowdoinham, ME) was placed on the white matter below the Purkinje cell layer. A recording electrode was moved along the Purkinje cell layer until spontaneous activity of a cell was detected. We unequivocally identified the Purkinje cell layer as the source of the detected current because moving the recording electrode along the molecular layer of the cerebellum resulted in an inversion of the response polarity (14).

Immunocytochemistry. Animals ($n = 5$ per group) were deeply anesthetized with pentobarbital (50 mg/kg) and perfused transcardially with 0.9% NaCl, followed by 400 ml of fixative (4% paraformaldehyde/0.1 M sodium phosphate, pH 7.4; PB). Brains were removed, postfixed 3 h in the same fixative at room temperature, and cryoprotected by immersion in 30% sucrose solution in PB at 4°C. Serial 30-μm-thick coronal frozen sections were cut in a cryostat and processed for immunocytochemistry. The sections were soaked in PB containing 10% methanol and 0.3% H_2O_2 for 30 min at room temperature to block endogenous peroxidase and then rinsed in PB. To visualize calbindin, sections were incubated overnight at 4°C with a monoclonal antibody (anti-calbindin, Sigma) diluted 1:1,000 in PB containing 0.1% bovine albumin, 3% nonimmune goat serum, and 0.2% Triton X-100. After several washes in PB, sections were incubated with a goat anti-mouse IgG horseradish peroxidase conjugate (Bio-Rad) diluted 1:100. The peroxidase activity was visualized by incubating the sections in PB containing 0.03% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.03% H_2O_2 for 10 min. For quantitative analysis, sections were observed through a ×10 objective in a Leitz microscope. The entire inferior olivary complex of each animal was evaluated (between 60 and 70 consecutive sections per animal). Calbindin-positive cells within a given section were counted, and the number of cells per section was expressed as the mean of all the sections obtained for each animal.

Biochemical Markers of Cerebellar Function. Calbindin and glutamate receptor subunit 1 levels in cerebellar extracts from the different groups of animals were measured by Western blot analysis as described elsewhere in detail (15) and expressed as percent of control levels. γ-Aminobutyric acid (GABA) and glutamate levels were assessed by HPLC as described (2). IGF-I in cerebellum and serum was determined with an specific radioimmunoassay (16).

RESULTS

Cerebellar Ataxia and IGF-I. Motor discoordination in 3AP-injected rats was evaluated with the rota-rod test. One week after 3AP injection, animals stayed in the rotating rod almost 10 times less (20.1 ± 13.3 sec, mean ± SEM) than

control rats (189 ± 16 sec; $F = 2.3$; $P < 0.0001$), indicating that their motor coordination was severely impaired. The rats remained ataxic for the duration of the study: 6 weeks after 3AP injection they showed an identical inability to stay in the rod (<20 sec).

Previous studies indicated a marked decrease in cerebellar IGF-I levels in ataxic animals (6). IGF-I acts as a trophic factor for Purkinje cells both *in vivo* and *in vitro* (2, 17). Hence, we evaluated several phenotypic markers of these cerebellar neurons to determine whether lower IGF-I levels due to cerebellar deafferentation would result in deleterious changes in Purkinje cells. We found that calbindin, GABA, and glutamate receptor subunit 1 levels were significantly decreased 2 weeks after injection of 3AP (Table 1). In accordance with previous observations, we found that glutamate and IGF-I levels in the cerebellum were also significantly depleted (Tables 1 and 2 and refs. 6 and 11).

Because recent findings show that serum IGF-I levels varied in parallel with cerebellar IGF-I levels and that peripheral IGF-I can be selectively taken up from the blood stream into the brain parenchyma (20), we also evaluated serum IGF-I levels in these animals and found a significant decrease in 3AP-injected rats (Table 2). This reinforces the use of 3AP-injected rats as a model of human cerebellar ataxia because in this disease a decrease in both serum IGF-I levels and cerebellar calbindin is found (5, 21).

To rule out a possible unspecific toxic action of 3AP on the liver, the main source of circulating IGF-I (22), we also evaluated the effect of an alternative way of lesioning the inferior olive and found that a similar decrease in IGF-I levels both in serum and cerebellum was present after ablation of this brainstem nucleus by electrocoagulation (Table 2).

Although injection of 3AP at the doses used renders the animals permanently ataxic, plastic rearrangements in the cerebellar cortex circuitry of these animals take place within 1–2 months of the 3AP injection (18). These poorly characterized events are modulated by endogenous IGF-I (7) and eventually lead to recovery of the spontaneous electrical activity of the Purkinje cell, even though these neurons are permanently deprived of their climbing fiber input (18). To determine whether there is a relationship between cerebellar plasticity and IGF-I levels, we measured levels of this trophic peptide and cerebellar cell markers 4 weeks after 3AP injection. Functional indicators of cerebellar function showed a striking recovery correlating with an increase in serum IGF-I levels (Tables 1 and 2, 4 weeks). Thus, recovery of Purkinje cell function, as determined by electrophysiological (18) and biochemical (Table 1) parameters, is paralleled by recovery of serum IGF-I levels (Table 2). Interestingly, IGF-I levels in cerebellum lagged behind serum levels and did not return to normal until 6 weeks after injection of 3AP (data not shown). Moreover, even though in this model of ataxia the animals recovered Purkinje cell function (18), they never recovered normal motor coordination.

Recovery from Ataxia by Administration of IGF-I. From these observations, we postulated that a lack of recovery of motor coordination in these animals was due to the fact that normalization of cerebellar IGF-I levels occurs too late to rescue any inferior olivary neurons. These neurons die within hours after injection of the neurotoxin (23). We reasoned that if we provided IGF-I before olivary neurons die, we might be able to rescue these neurons. Thus, a second group of rats was injected with 3AP, implanted simultaneously with osmotic minipumps containing either IGF-I or vehicle, and connected to an intracerebroventricular (i.c.v.) cannula. Continued evaluation of the motor coordination in these rats showed that ataxic animals receiving IGF-I recovered gradually throughout time. Although for the first 5 days of treatment 3AP-injected animals were still ataxic, by day 10 they started to recover and gained full motor coordination after 4 weeks (Fig. 1). A continuous supply of IGF-I for more than 3 weeks was necessary because interruption of the treatment at day 15 resulted in blockade of further recovery (data not shown). Vehicle-injected 3AP animals remained ataxic throughout the duration of the study.

Motor ability of IGF-I-treated ataxic animals was further evaluated by using an additional test of motor performance. As shown in Fig. 1*B*, ataxic animals receiving IGF-I and control animals stayed on a platform that was tilted to a similar degree of inclination and that was significantly steeper than the degree of inclination resisted by vehicle-treated ataxic rats ($F = 3.5$; $P < 0.001$). Hence, we concluded that full recovery of motor coordination may be achieved when IGF-I levels are therapeutically increased at the same time that olivary neurons are degenerating.

Previous findings in human cerebellar ataxia and observations in several animal models support the idea of a biologically significant cross-talk between the peripheral (endocrine) and central (paracrine/autocrine) IGF-I trophic system (5, 20). By assuming the hypothesis that serum IGF-I levels are directly influencing brain IGF-I levels, we administered IGF-I by a peripheral route with the hope of finding an easy way to treat human ataxia. Indeed, the recovery elicited in 3AP rats by subcutaneous (s.c.) administration of IGF-I was comparable to that obtained after central delivery of the peptide (Fig. 1). Thus, regardless of the route used for administration of IGF-I to the rats, both cerebellar and serum IGF-I levels were greatly increased (Table 2). Although the increase in serum IGF-I levels after i.c.v. administration may be accounted for by the transport from the cerebrospinal fluid to the circulation, increased cerebellar levels of IGF-I after s.c. administration of the peptide may be explained by specific uptake of IGF-I by the brain (20). Hence, we conclude that peripherally administered IGF-I is able to reach the brain parenchyma in sufficient amounts to induce functional recovery. Alternatively, elevated blood IGF-I levels may regulate the brain IGF-I system by as yet undetermined mechanisms that eventually result in increased brain IGF-I. At any rate, these findings open the

Table 1. Indicators of cerebellar function in 3AP ataxic rats

	Calbindin		Glur1		GABA		Glutamate	
	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks	2 weeks	4 weeks
Control	100 ± 8	100 ± 6	100 ± 5	100 ± 6	5.8 ± 0.6	5.7 ± 0.7	19.9 ± 0.9	19.6 ± 0.7
3AP	37.6 ± 12*	101 ± 10	42.6 ± 9.2*	99.3 ± 8.3	3.8 ± 0.3*	5.4 ± 0.5	11.4 ± 1.5*	18.6 ± 2

Levels (% of control for calbindin and Glur1 and ng/mg protein for GABA and glutamate) of the various markers showed that animals injected with 3AP have a generalized depressed cerebellar function 2 weeks after injection of the neurotoxin. Although 3AP rats showed no signs of behavioral recovery throughout the time of the study (see Fig. 1), the levels of the various cerebellar indicators return to normal after 4 weeks of the injection of the neurotoxin. This time course of recovery parallels that reported for normalization of the spontaneous activity of Purkinje cells, which is attributed to plastic rearrangements of cerebellar circuitries (18). Calbindin and glutamate receptor subunit 1 (GluR1) are specific markers for Purkinje cells in the cerebellum (19), and GABA and glutamate are used as the major phenotypic markers of cerebellar neurons, including Purkinje cells (GABAergic), granule cells (glutamatergic), and the cerebellar interneurons (GABAergic). *, $P < 0.01$ vs. control, as determined by Student's *t* test.

Table 2. Levels of IGF-I in rodent models of cerebellar ataxia

Treatment	Cerebellum IGF-I pg/mg protein		Serum IGF-I ng/ml serum	
	2 weeks	4 weeks	2 weeks	4 weeks
Control	670.3 ± 10.2	672.5 ± 10	142.2 ± 1.6	140 ± 2.8
3AP	440.8 ± 10.2**	500.6 ± 10.2**	112.6 ± 5.1***	131.6 ± 3.4
Control EC	502.9 ± 19.8	ND	164.8 ± 4.9	ND
EC	270.2 ± 10.8**	ND	129.6 ± 8.2**	ND
3AP + IGF-I (i.c.v.)	ND	3038.2 ± 166.1*	ND	446.9 ± 33.7*
3AP + IGF-I (s.c.)	ND	1320 ± 150.8***	ND	452.5 ± 38.6*

Peripheral (serum) and local (cerebellum) IGF-I levels changed in parallel after induction of ataxia. During early phases of the ataxic process (first 2 weeks), IGF-I levels in both compartments are depressed, but after a month of the injection of 3AP IGF-I levels tend to recover. Ataxic animals showed changes in IGF-I levels regardless of the method employed to elicit ataxia (neurotoxin or electrolytic lesion). As expected, infusion of IGF-I by either s.c. or i.c.v. osmotic minipumps resulted in significantly increased IGF-I levels both in cerebellum and in serum. These results suggest that decreased serum IGF-I may be related to olivo-cerebellar dysfunction because in human olivo-ponto-cerebellar atrophy similar decreases are found (5). *, $P < 0.001$; **, $P < 0.01$; ***, $P < 0.05$ vs. respective controls (Student's t test). ND, not determined; EC, electrocoagulation.

possibility of treating human ataxia with subcutaneous administration of IGF-I.

Because pilot studies in ataxic rats indicated that the ability to recover motor coordination depended on the number of surviving inferior olive neurons and this, in turn, depended on the dose of 3AP used, we hypothesized that the recovery elicited by IGF-I was due to its ability to rescue substantial numbers of climbing fiber axons by both promoting increased neuronal survival and inducing axon sprouting (24). Thus, we determined the integrity of the olivocerebellar pathway of IGF-I-treated ataxic rats by both electrophysiological and morphological analyses. Evaluation of the climbing fiber input to the cerebellar cortex of these rats indicated that 3AP-injected animals treated with IGF-I showed a normal pattern of activation of the cerebellar cortex by climbing fiber afferents but, in vehicle-treated 3AP animals, this input was absent (Fig. 2). Similarly, analysis of the number of neurons in IGF-I-treated animals showed a survival of 92% of calbindin-positive cells in the inferior olive nucleus, the source of climbing fiber afferents to the cerebellar cortex, but in vehicle-treated 3AP animals, less than 20% of these neurons remained alive ($F = 2.6$; $P < 0.001$; Fig. 3).

We also evaluated the efficacy of delayed IGF-I treatment of ataxic animals to determine whether recovery of function is possible even after severe neuronal loss. Osmotic minipumps filled with IGF-I (1 mg/ml) were implanted (s.c.) in rats that had been injected with 3AP 5 days earlier. Delayed treatment with IGF-I also resulted in full behavioral recovery as determined by the rota-rod test: controls, 299 ± 0.5 sec; vehicle-treated ataxic rats, 2.7 ± 0.5 sec; IGF-I-treated ataxic rats, 292 ± 0.7 sec (day 30 of treatment, $P < 0.001$ vs. vehicle-treated ataxic rats, Student's t test). Importantly, the number of neurons rescued by delayed IGF-I treatment represented only 27 ± 8% of the total inferior olive population. These results strengthen the therapeutical potential of IGF-I because even after severe neuronal depletion it still promotes functional recovery.

DISCUSSION

Our results show that full behavioral recovery can be achieved after peripheral administration of IGF-I in a rat model of human olivo-cerebellar degeneration. Complete recovery occurred in parallel with normalization of olivo-cerebellar func-

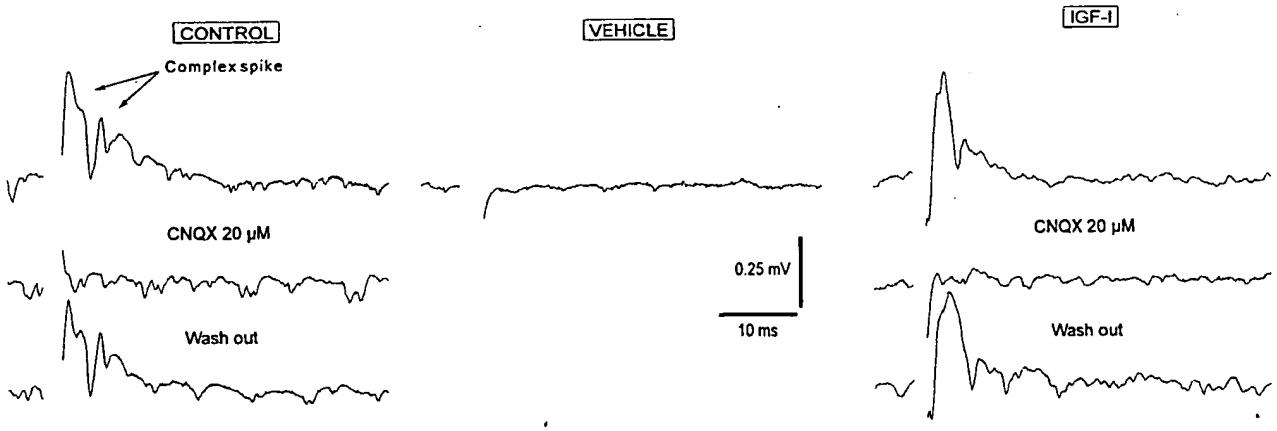

FIG. 2. Treatment of ataxic rats with IGF-I results in maintenance of the climbing fiber input to Purkinje cells. Electrophysiological recordings were made from cerebellar slices of control rats (Control traces), rats injected with 3AP and treated with IGF-I (IGF-I traces), and 3AP rats receiving a constant infusion of saline (Vehicle traces). Typical all-or-none climbing fibers responses (complex spikes) were readily obtained in both controls (91 of 100 trials, $n = 7$) and IGF-I-injected ataxic rats (82 of 100, $n = 7$) using a moderate stimulation intensity (range of 10–40 nA for all experimental groups). Synaptic responses were completely abolished by using the selective non-*N*-methyl-D-aspartate glutamate receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM, Alexis, Laufelfingen, Switzerland) and recovered after wash out. When the same experimental procedure was used in the vehicle group, synaptic responses were very rarely obtained (28 of 100, $n = 7$) even under stronger stimulation intensity (up to 70 nA). The latter agrees with previous results in rats deafferented by either 3AP or electrocoagulation of the inferior olive where a loss of the climbing fibers response is found (18, 25). Results shown for the group treated with IGF-I correspond to a representative 3AP animal receiving IGF-I s.c.

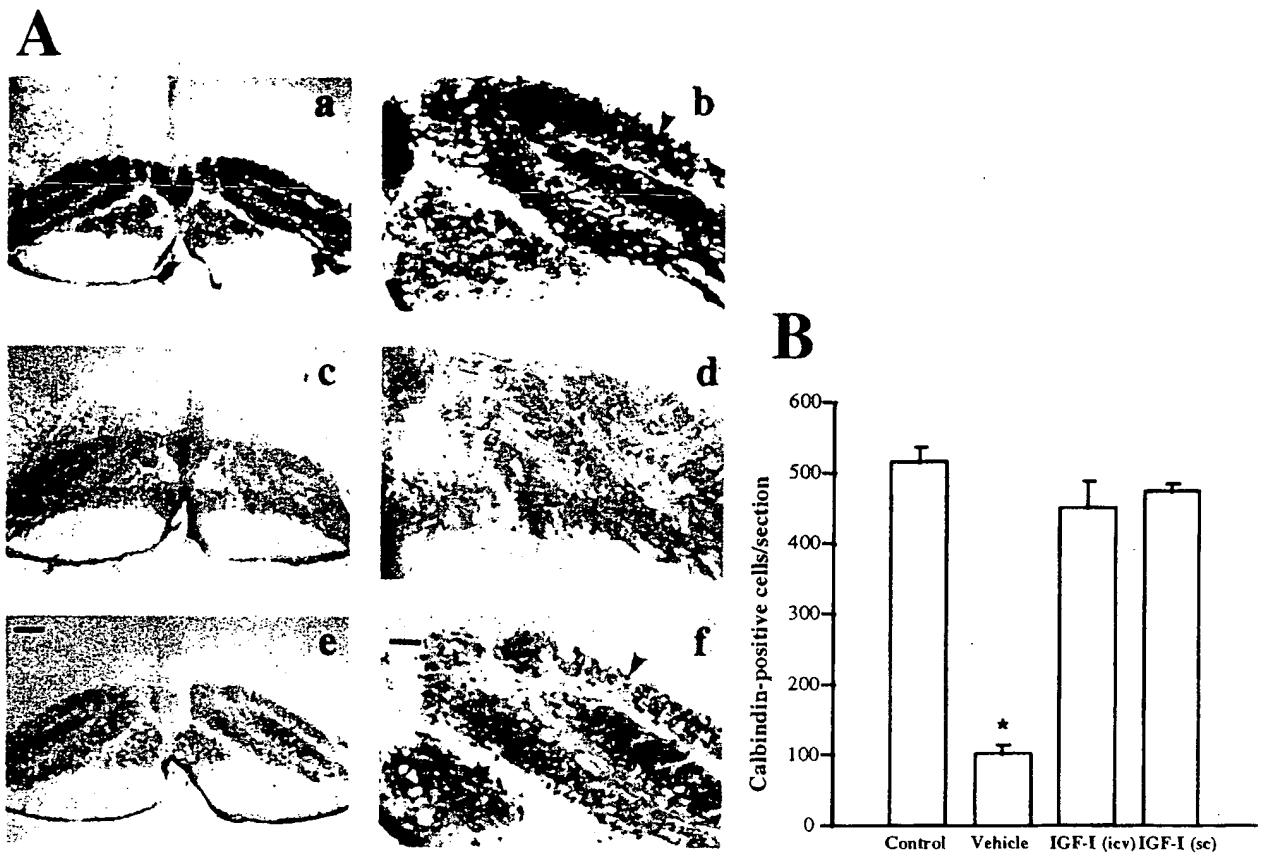

FIG. 3. IGF-I treatment of ataxic rats resulted in greatly increased survival of inferior olive neurons. (*A*) Representative photomicrographs of inferior olive neurons stained with calbindin, a specific marker of these neurons (26) from control (*a* and *b*), vehicle-treated 3AP rats (*c* and *d*), and IGF-I-treated 3AP rats (*e* and *f*). Note that vehicle-treated 3AP rats have almost no surviving neurons but those treated with IGF-I have almost as much as intact animals. Cresyl violet staining confirmed neurodegeneration and gliosis of the inferior olive nuclei after 3AP injection, as reported (6, 11). (*a*, *c*, and *e*) General overview of the inferior olive nuclei. (Bar = 250 μm.) (*b*, *d*, and *f*) Higher magnification view of inferior olive neurons. (Bar = 50 μm.) Arrowheads show calbindin-positive neuronal bodies. (*B*) Quantification of neurons in each group showed that IGF-I-treated ataxic rats have significantly greater number of inferior olive neurons compared with vehicle-treated 3AP animals and almost as many as control animals. *, $P < 0.001$ vs. all other groups. Data are the number of calbindin-positive cells per section (mean ± SEM).

tion. It is noteworthy that neuronal death is usually a protacted process spanning many years in humans (27). Further, the present results show that IGF-I treatment restored full motor function even in ataxic animals with severe neuronal depletion. Hence, a potential therapeutic use of IGF-I in at least a selected subset of human neurodegenerative diseases affecting cerebellar function is supported by these findings.

IGF-I is just one of the many neurotrophic substances known. Previous characterization of its trophic activities in the cerebellar cortex during development and in the adult (2, 3, 15, 17) allowed us to hypothesize its involvement in the pathogenesis of cerebellar degeneration leading to ataxia (5). Several other models of cerebellar ataxia are currently used in addition to the 3AP model used in this study. These include different types of mouse neurological mutants showing various degrees of neuronal loss and affecting different subsets of cerebellar and cerebellar-related pathways (11). Cerebellar degeneration in humans also includes a variety of different conditions and syndromes of diverse pathogenesis such as Friedreich ataxia, spontaneous cerebellar cortical degeneration, or Wernicke–Korsakoff syndrome. Most of these diseases show a very wide range of age of onset and duration and frequently develop over the course of years. Although the pathogenesis of these types of syndromes is most likely multifactorial, it ultimately results in insults to specific populations of neurons within motor pathways (28). Thus, any reparative agent, such as IGF-I may help injured neurons to recover.

Although the mechanisms underlying the trophic effects of IGF-I on injured inferior olive neurons are unknown, Purkinje cells express IGF-I during their entire lifespan, with prominent expression at times coincident with the establishment of the olivo-cerebellar pathway (2, 29). Thus, IGF-I may be a target-derived trophic factor for inferior olive neurons not only during development but also during adulthood. At any rate, the ability of IGF-I to rescue inferior olive neurons is remarkable in view of the fact that IGF-I is also a survival factor for developing Purkinje neurons, the postsynaptic targets of inferior olive neurons (15), and for immature cerebellar granule cells (30). Because an IGF-I trophic circuitry linking the inferior olive and cerebellar cortex and encompassing the entire cerebellar motor pathway has been described (1, 17), the present observation may help explain the biological significance of this trophic circuitry: a single neurotrophic factor may contribute to the maintenance of an entire functional circuitry. Although further work is needed to ascertain this possibility, these data suggest that IGF-I promotes the survival of interconnected populations of neurons within cerebellar circuitries.

It is worth noting that although the number of patients with the different types of cerebellar ataxia fortunately do not reach the devastating proportion of other neurological illnesses such as Alzheimer or Parkinson's disease, the complete lack of even paliative treatments for this ailment makes it of significant societal interest. IGF-I is currently being tested as a therapeutical aid in diseases such as Laron dwarfism, diabetic neuropathy, and amyotrophic lateral sclerosis (8, 31, 32). Thus, its possible use in the treatment of human cerebellar ataxia appears of great promise.

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